Journal of Steroid Biochemistry & Molecular Biology 158 (2016) 46–55

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Expression and functional roles of estrogen receptor GPR30 in human intervertebral disc

Aiqun Wei[a], Bojiang Shen[a], Lisa A. Williams[a], Divya Bhargav[a], Feng Yan[b], Beng H. Chong[b], Ashish D. Diwan[a,*]

[a] Department of Orthopedic Research, Orthopedic Research Institute, St George Hospital, University of New South Wales, Sydney, Australia
[b] Department of Hematology, St George Hospital, University of New South Wales, Sydney, Australia

ARTICLE INFO

Article history:
Received 1 July 2015
Received in revised form 21 January 2016
Accepted 22 January 2016
Available online 24 January 2016

Keywords:
Intervertebral disc
Estrogen receptor
GPR30
GPER
Nucleus pulposus

ABSTRACT

Estrogen withdrawal, a characteristic of female aging, is associated with age-related intervertebral disc (IVD) degeneration. The function of estrogen is mediated by two classic nuclear receptors, estrogen receptor (ER)-α and -β, and a membrane bound G-protein-coupled receptor 30 (GPR30). To date, the expression and function of GPR30 in human spine is poorly understood. This study aimed to evaluate GPR30 expression in IVD, and its role in estrogen-related regulation of proliferation and apoptosis of disc nucleus pulposus (NP) cells. GPR30 expression was examined in 30 human adult NP and 9 fetal IVD. Results showed that GPR30 was expressed in NP cells at both mRNA and protein levels. In human fetal IVD, GPR30 protein was expressed in the NP at 12–14 weeks gestation, but was undetectable at 8–11 weeks. The effect of 17β-estradiol (E2) on GPR30-mediated proliferation and interleukin-1β (IL-1β)-induced apoptosis of NP cells was investigated. Cultured NP cells were treated with or without E2, GPR30 antagonist G36, and ER antagonist ICI 182,780. NP cell viability was tested by MTS assay. Apoptosis was determined by flow cytometry using fluorescence labeled annexin-V, TUNEL assay and immunocytochemical staining of activated caspase-3. E2 enhanced cell proliferation and prevented IL-1β-induced cell death, but the effect was partially blocked by G36 and completely abrogated by a combination of ICI 182,780 and G36. This study demonstrates that GPR30 is expressed in human IVD to transmit signals triggering E2-induced NP cell proliferation and protecting against IL-1β-induced apoptosis. The effects of E2 on NP cells require both GPR30 and classic estrogen receptors.

1. Introduction

Intervertebral disc (IVD) degeneration is one of the leading causes of chronic back pain, with both a high prevalence and associated disability in a recent global burden of disease study [1]. Characterized by a loss of cells and extracellular matrix leading to disc height reduction [2,3], IVD degeneration is driven at least in part by aging [4]. Collagens and proteoglycans appear to undergo specific age-related changes which influence matrix quality and integrity or alter cellular reparative pathways, potentially contributing to degeneration [4,5].

Pathological apoptosis has been shown to be a key factor responsible for the decrease in cell number in nucleus pulposus (NP) during disc degeneration [6]. Consequently, prevention of NP cell apoptosis could slow down the progression of IVD degeneration. 17β-estradiol (E2) has been shown to promote human annulus fibrosus (AF) cell proliferation [7], and to exert anti-apoptotic effects in rat disc cells [8,9]. However, the anti-apoptotic effect of E2 in human NP cells remains unknown.

Estrogen exerts a range of biological effects in a variety of tissues, including the musculoskeletal system and connective tissues [10,11]. Estrogen also plays an important role in the regulation and maintenance of healthy spinal discs in women [12]. Withdrawal of estrogen at menopause has a negative effect on the structure of bone and IVD. In particular, elderly females experience a faster rate of lumbar disc degeneration with greater severity than age matched men, despite the frequency of lumbar disc degeneration being higher in young and middle-aged men than in women [13,14]. Further, degenerative lumbar spondylolisthesis, most common at the L4/L5 level, was nearly threefold higher in

* Corresponding author at: Orthopedic Research Institute and Department of Orthopedic Surgery, St George Hospital, Level 2, 4-10 South Street, Kogarah NSW 2217, Australia. Fax: +61 2 8566 7177.
E-mail addresses: a.diwan@spine-service.org, a.diwan@unsw.edu.au (A.D. Diwan).

http://dx.doi.org/10.1016/j.jsbmb.2016.01.012

women with a history of ovariectomy compared to non-ovariectomized controls [15]. Indeed, ovariectomy caused lumbar IVD degeneration in a rodent model [16]. Estrogen replacement therapy (ERT) has beneficial effects in maintaining disc height, an indicator of disc degeneration, where estrogen-repleted women maintained higher disc heights than untreated post-menopausal women [17,18].

G protein-coupled receptor 30 (GPR30), a 7 transmembrane spanning G protein-coupled receptor with high affinity for estrogen [19], was localized at the cell membrane, in subcellular compartments and within the nucleus of normal or cancer cells, involving in estrogen evoked physiological and pathological events in central nervous, immune, renal, reproductive and cardiovascular systems [20,21]. GPR30-mediated estrogenic signaling was shown to activate multiple intracellular signaling pathways including mobilization of intracellular calcium and activation of protein–lipid kinase pathways [22,23]. Its presence has been touted to facilitate rapid non-genomic signaling events to result in cell migration, survival and proliferation that are independent of classic estrogen receptor α or β (ERα or ERβ) in a variety of normal and malignant cell types [11,22,24]. More recently, genomic signaling mechanism of GPR30 has been identified as the receptor nuclear translocation in an importin-dependent manner [21].

The responsiveness to estrogen of a given tissue is determined by the expression of ER and GPR30, their co-regulator expression and the signaling interplay between receptors [25]. Expression of both ERα and ERβ has been detected in human disc NP and AF, with significantly decreased expression concomitant with the aggravation of IVD degeneration [7,26]. However, GPR30 expression and its physiological role in relation to estrogen function in IVD remains unclear. In this study, we aimed to investigate whether GPR30 was expressed in the NP tissue of human IVD at various stages of life, and if so whether it plays a role in transmitting E2 signals for cell proliferation and survival, both aspects relevant to disc regeneration and health.

2. Materials and methods

2.1. Tissue collection

The human NP tissues were freshly collected from elective surgeries following informed consent under ethical approval from the South-Eastern Health Service Human Research Committee, Sydney, Australia. A total of 37 disc samples were collected from 30 patients, in total of 9 female and 21 male samples with a median age of 41 ± 15 and 39 ± 14 respectively. Nine of the 37 disc samples were obtained from 6 scoliotic patients (age 13–31, 23 ± 7, 2 female and 3 male). The other 28 disc samples were obtained from 24 patients (age 27–79, 46 ± 13, 7 female and 17 male) with moderate signs of degeneration, including decreased water content and disc height, as determined by magnetic resonance imaging (MRI) and X-ray. Seven of the 37 samples were used for cell culture experiments, and 30 of the samples were used for both histology and RNA analysis.

The spinal columns of 9 human fetuses at 8–14 weeks gestation were obtained at termination of pregnancy with ethical approval from the Human Care and Ethics Review Committee of the University of New South Wales. No genetic abnormalities were reported for these specimens. The sex of each sample was not provided. Tissue was freshly collected and immediately fixed in 10% neutral buffered formalin for 24 h followed by sectioning in the sagittal plane and embedding in paraffin. Details of tissue analyses in this study are provided in Table 1.

Table 1
Percentage of GPR30 mRNA and protein expression detected in NP samples.

	GPR30 positive samples (%)	
	RT-PCR (n = 12)	IHC (n = 30)
Adult tissue		
Female	80% (4/5)	78% (7/9)
Male	71% (5/7)	67% (14/21)
Total	75% (9/12)	79% (21/30)
Foetal tissue		
8–11 Weeks	n/a	0% (0/4)
12–14 Weeks	n/a	80% (4/5)

IHC = Immunohistochemistry.

2.2. RNA extraction and RT-PCR

Freshly collected NP tissues were ground to powder in liquid nitrogen using a mortar and pestle. Total RNA from tissues was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA) and from cultured cells using H-RNA purification kits (Roche Diagnostics, Manheim, Germany) according to manufacturers' instructions. The cDNA was generated by reverse transcription of 1 μg of total RNA using SuperScript III first-strand synthesis kit (Life Technologies). The 1:40 diluted cDNA was used in 20 μl reactions for PCR analysis using a Rotor-Gene RG3000 system (Corbett Life Science, Sydney). The thermal profile for all reactions was as follows: 5 min at 95 °C, followed by 40 amplification cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C [22]. The primers were designed based on published mRNA sequences using the Primer3 online tool. GPR30 (GenBank accession no. NM_001505): forward primer: 5-CTCTTCCCCATCGGCTTTGT-3 and reverse primer: 5-TACAGGTCGGGGATGGTCAT-3. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession no. NM_001034034.1): forward primer: 5-GAGTCCACTGGGGTCTTCACT-3; and reverse primer: 5-GCGTGGACAGTGGTCATAAGTC-3. The PCR end products of GPR30 and GAPDH were analyzed by electrophoresis in 10% polyacrylamide gels and the DNA bands were visualized with ethidium bromide.

2.3. Immunohistochemical and immunofluorescence staining

Paraffin embedded blocks of tissue were cut into sections (4 μm) and mounted on superfrost plus slides (Thermo Fisher, Australia). The slides were deparaffinized and hydrated through a graded ethanol series. After antigen retrieval, endogenous peroxidases were scavenged with 3% (v/v) H_2O_2 and non-specific binding was blocked by incubation in 10% skim milk in Tris-HCl buffer. The slides were then probed with a primary rabbit anti-GPR30 polyclonal antibody (1:150, Santcruz, Australia). Thereafter, the slides were washed and treated with multilink solution (DAKO, Sydney, Australia) followed by streptavidin-conjugated peroxidase incubation. The sections were visualized with a 3, 3′-diaminobenzidine hydrochloride solution (DAB, DAKO) and counterstained in haematoxylin. The primary antibody was omitted for a negative control. The images were captured using a Leica DMLB microscope.

Immunofluorescence staining was performed on cells cultured on glass coverslips following fixation with 4% paraformaldehyde. Cells were stained with or without a permeabilization step with 0.1% Triton X-100, blocked in 5% normal goat serum. Primary polyclonal rabbit antibody against GPR30 (Santcruz) was diluted 1:200 before applying to slides, followed by incubating with Alexa-488 conjugated anti-rabbit IgG secondary antibody in 1:500 dilution. The slides were counterstained with a 500 nM of propidium iodide (PI, Sigma). Finally, mounted coverslips were visualized under a laser scanning confocal fluorescence microscope (Olympus Sverige AB, Solna, Sweden).

2.4. Cell culture and treatment

Freshly obtained NP tissues were immediately subjected to 0.025% collagenase digestion overnight. Subsequent primary cell cultures were grown in a complete medium containing DMEM (Life Technologies), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin at 37 °C, 5% CO_2/95% air until reaching confluence. All experiments were conducted using cell cultures at passage 1–2.

For experiments, cells were seeded in DMEM medium with 1% FBS overnight. Cells were then pre-treated with GPR30 antagonist G36 (250 nM), and/or the estrogen receptor (ERα and ERβ) antagonist ICI 182,780 (200 nM) for 1 h prior to treatment with E2 (50–100 nM) for 48 h. The cells in the control groups were incubated for equivalent periods of time without further treatment. For immunocytochemistry and immunofluorescence staining, cells were sub-cultured at a concentration of 5×10^4/ml in 6 well plates with glass cover slips for 2 days before assay.

2.5. Cell proliferation

Cell survival was measured with the MTS cell proliferation assay kit (Promega, Madison, WI) using 1×10^4 cells/well in 96-well plates. To observe the effect of estrogen on NP cell proliferation, different concentrations of E2 (50–100 ng/ml) were applied to the cultures. Assays were performed as specified by the manufacturer, such that only viable cells are able to metabolically reduce tetrazolium salts to formazan salts, detected directly in a spectrophotometer at 490 nm.

2.6. Apoptosis detection

2.6.1. TUNEL assay

The effect of E2 on NP cell apoptosis was further examined using DeadEnd™ fluorometric TUNEL kit (Promega Corporation, Madison, USA,) as per the manufacturer's instruction. Briefly, NP cells were cultured on coverslips and then apoptosis was induced using interleukin-1β (IL-1β) (R&D Systems, Minneapolis, MN) at 20 ng/ml in serum-free culture condition for 48 h. The cells were fixed in 4% paraformaldehyde and treated with permeabilization solution consisting of 0.1% Triton X-100 for 20 min. After washing, the cells were incubated with labeling solution containing terminal deoxynucleotidyl transferase and fluorescein dUTP for 2 h. Cells were then washed and double stained with 1 μg/ml DAPI for 10 min. The labeled NP cells were examined under a fluorescence microscope (Olympus, Germany). To quantify apoptotic cells, the TUNEL positive cells in 3 independent cultures were counted in 5 replicates of 5 randomly selected fields. The green florescent stains are TUNEL positive cells and blue nuclei staining represents the total number of cells. The proportion of TUNEL positive cells was expressed as a percentage of the total cells.

2.6.2. Flow cytometry

The population of induced apoptotic cells was determined using the Alexa Fluor-488 conjugated annexin-V cell death detection kit (Life Technologies) as specified by the manufacturer. Briefly, IL-1β apoptotic induction was performed with 20 ng/ml for 48 h. E2 at a concentration of 50 nM was added in cultures and remained in the medium during IL-1β treatment. Either G36 or ICI 182,780 or a combination of both was pre-administrated in cultures for 0.5 h in corresponding co-treatment groups. After incubation for 48 h, cells were harvested and incubated with the Alexa Fluor-488 annexin-V in binding buffer at room temperature for 15 min in the dark. The cells were measured by a flow cytometer—FACSCanto II and the data were analyzed with FACSDiva software (BD Biosciences, CA, USA).

2.6.3. Activated caspase-3 by immunocytochemistry

Activated caspase-3 was detected by immunocytochemical staining. Briefly, NP cells were subcultured on glass coverslips and fixed with 4% paraformaldehyde, followed by blocking of nonspecific binding with a 10% skim milk solution. Primary mouse anti-cleaved caspase-3 antibody (1:100, Cell Signaling Technology) was added to each coverslip, incubated at 4 °C for 12 h. After several washes with PBS, cells were incubated using multilink solution and visualized with 3,30-diaminobenzidine hydrochloride solution (DAKO).

2.7. Statistical analysis

Data analysis of multiple experimental groups was performed with one way analysis of variance (ANOVA) followed by Duncan's test using SigmaStat software (SPSS, Inc., San Rafael, CA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. GPR30 is expressed in human NP tissue and cultured NP cells

GPR30 mRNA expression was detected in 4 out of 5 male and 5 out of 7 female adult NP tissue samples (Fig. 1, Table 1). Immunohistochemical staining identified positive GPR30 protein

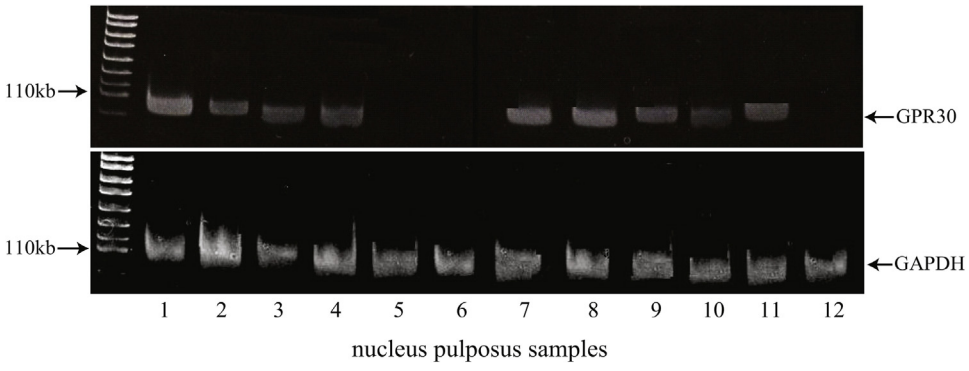

Fig. 1. Expression of GPR30 mRNA in the nucleus pulposus of human intervertebral disc. RT-PCR detection of GPR30 mRNA levels in 12 human NP samples. The gel image shows the PCR products of GPR30 (top panel) and GAPDH (bottom panel) separated on a DNA-retardation gel of 10% polyacrylamide. Lanes 1–12 represent the 12 NP samples in the representative image ($n = 3$).

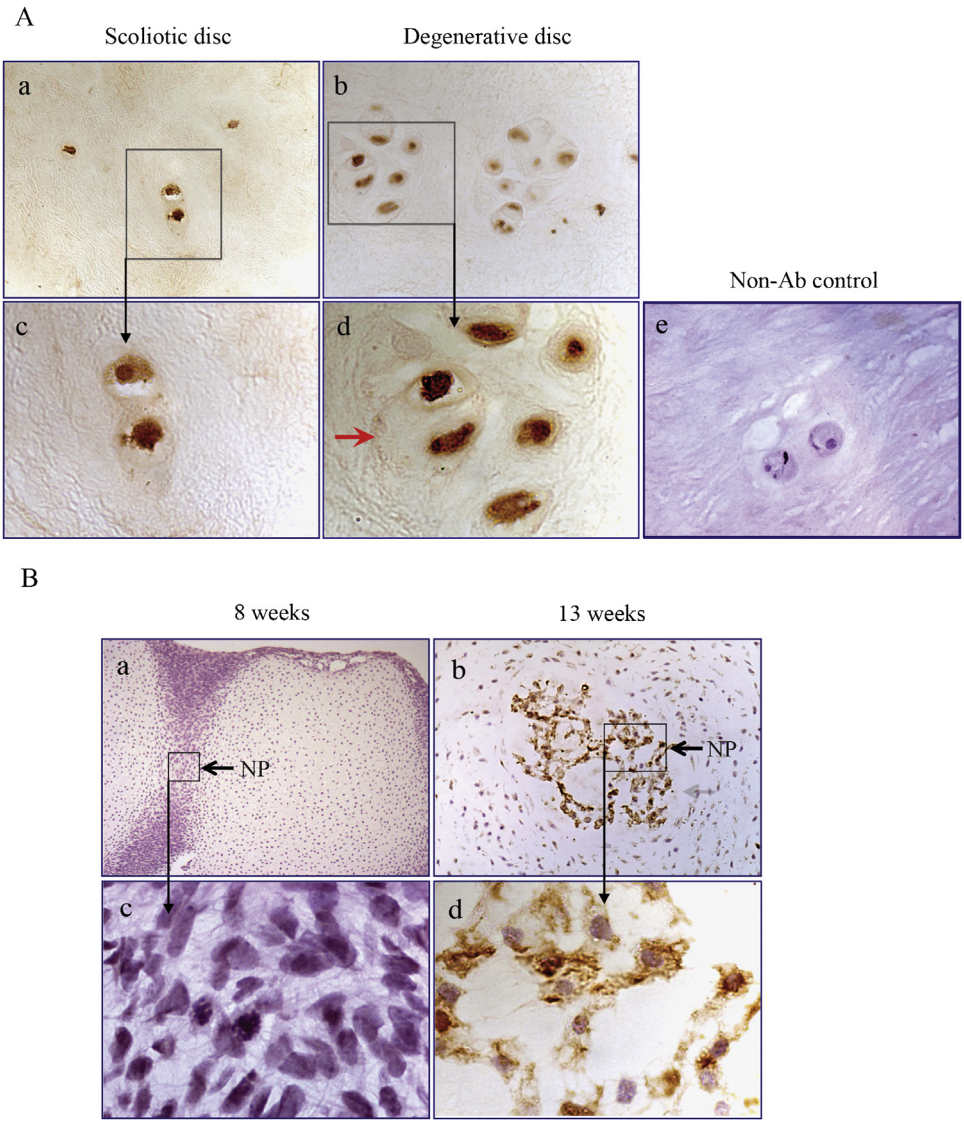

Fig. 2. (A) representative images of GPR30 immunohistochemical localisation in human nucleus pulposus tissues. Images show NP tissue stained with GPR30 specific antibody at low magnification (a and b) and the boxed regions at higher magnification (c and d). Strong expression of GPR30 associated with the cell nucleus region is visible in tissue samples derived from both scoliotic (a and c), and degenerative discs with proliferative cell clusters (b and d). Weaker staining is shown towards the cell periphery at the NP cell plasma membrane (d arrow). No GPR30 expression was observed in the negative controls where the first antibody was omitted (e). Original magnification: a, b and e ×20; c and d ×100. (B) GPR30 immunohistochemical localization in human fetal NP tissue. Images show specific GPR30 antibody staining of fetal spinal tissue sections at 8 weeks (a and c) and 13 weeks (b and d) gestation. Tissues are shown at low magnification (a and b, ×10) with boxed regions at higher magnification (c ×100, d ×40).

expression in 21 out of 30 adult NP tissue samples, in which 7 out of 9 were from female tissues and 14 out of 21 were male (Table 1). There was no significant difference in the GPR30 expression pattern in tissues derived from either degenerated or scoliotic tissues (Fig. 2A). Strong GPR30 protein expression was predominantly localized in the nucleus in all of the samples (Fig. 2A a–d), but was also detected in the plasma membrane of some NP cells (Fig. 2A a–d, arrow). No antibody staining was detected in the NP cells of the negative control (Fig. 2A e).

In human fetal tissue, GPR30 protein was detected in the developing nucleus pulposus of the disc. Strong brown staining was visible in NP cells in specimens from 12 to 14 weeks gestation (Fig. 2B b and d) but not at earlier time points between 8 and 11 weeks gestation (Fig. 2B a and c). Positive staining was visible in the cytoplasm of notochordal cells in the NP region, but most nuclei appeared to be stained blue with haematoxylin, lacking GPR30 antibody binding (Fig. 2B d). Staining did appear to be present at the plasma membrane in cells of the NP, with intracellular staining concentrated in regions near the nucleus (Fig. 2B b and d).

To further investigate the localization of GPR30 in NP cells, immunofluorescent staining was performed and analyzed by confocal microscopy. Consistent with the aforementioned observations in immunohistochemical staining, immunofluorescent staining confirmed that GPR30 protein expression was present in all cultured NP cells (Fig. 3). GPR30 expression on Triton X-100-permeabilized cells was found to have more extensive distribution across the cell, and was concentrated in the region of the nucleus (Fig. 3 a–f), including extrusions of the cellular membrane (Fig. 3 d and f). By comparison, intact cells showed a more restricted, punctate staining pattern that failed to outline the cell (Fig. 3 g–i). No GPR30 expression was detected in the negative controls lacking primary antibody (not shown).

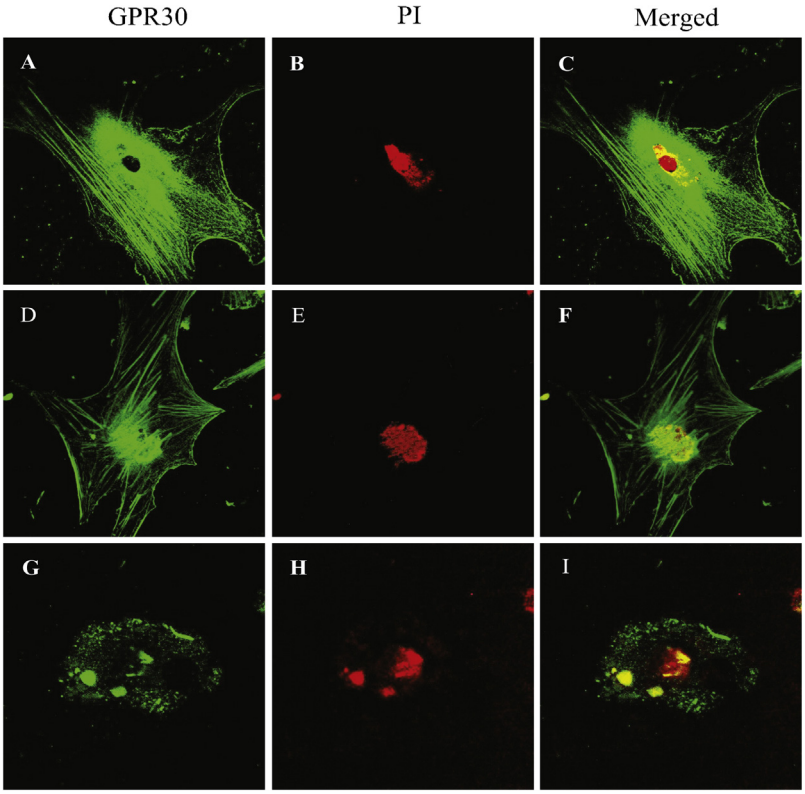

Fig. 3. Confocal microscopic images of GPR30 immunofluorescence in cultured human nucleus pulposus cells. Representative images show expression of GPR30 (green) on cultured NP cells either Triton X-100-permeabilized (A–F) or intact (G–I). Nuclei are stained using propidium iodide (PI, red). Co-localization of GPR30 with PI is observed when images merge (yellow; C, F, I) (original magnification: ×600). Images represent three independent tissue samples cultured in duplicate, with individual experiments performed in triplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. GPR30 blockage partially inhibits estrogen enhanced NP cell proliferation

The data from MTS assay showed that E2 could stimulate NP cell proliferation. To observe if GPR30 signaling is required in the E2 induced cell proliferation, NP cell cultures were stimulated with increasing concentrations of E2 (0–100 nM) with or without specific estrogen receptor antagonists G36 (for GPR30) or ICI 182,780 (for ERα/ERβ). Initial titration experiments showed peak stimulation at 50 nM E2, reaching a plateau at 100 nM with an approximately 1.5 fold increase when compared to control (data not shown). Further experiments were performed using 50 nM of E2 stimulation. NP cell proliferation was found to increase by up to 52% after 48 h exposure to E2 when compared with control ($p < 0.05$) (Fig. 4). Addition of either G36 or ICI 182,780 to NP cell cultures alone had marginal, but not significant effect on cell proliferation. Co-treatment with either G36 or ICI 182,780 and E2 resulted in partial blockage of E2-stimulated proliferation. Furthermore, treatment with a combination of G36 and ICI 182,780 completely inhibited E2stimulated NP cell proliferation ($p < 0.05$) (Fig. 4), indicating the involvement of both ERs and GPR30 in E2 mediated signal transduction.

3.3. GPR30 blockage partially reverses estrogen inhibited NP cell apoptosis

To investigate the potential role of E2 in protecting cells from IL-1β-induced apoptosis and the involvement of GPR30, we first utilized the TUNEL assay, analyzing DNA fragmentation. IL-1β treatment of NP cells for 48 h significantly increased the population of cells with positive TUNEL staining by 39% compared to control ($p < 0.01$, Fig. 5A, B). E2 appeared to exert a protective effect on IL-1β induced NP cell damage ($p < 0.05$), with fewer cells showing positive TUNEL staining (Fig. 5A). Co-treatment with either G36 or ICI 182,780 alone did not significantly block the protective effect of E2. However, the E2 effect was completely abolished by co-treatment with both G36 and ICI 182,780 (Fig. 5A and B).

The involvement of GPR30 in E2 signaling was further assessed using annexin-V staining and then analyzed by flow cytometry. IL-1β treatment for 48 h increased the number of apoptotic NP cells staining positive for annexin-V in culture to 29%, compared to 3% in the control (Fig. 6). E2 decreased the percentage of apoptotic cells to 8%, an approximately 2.5 fold decrease compared to IL-1β treatment alone (29%) (Fig. 6), indicating that E2 is able to reduce IL-1β-induced apoptosis in NP cells. Treatment in the presence of GPR30 antagonist G36 produced a partial reduction of E2 effect, with 12% apoptotic cells detected. The protective effect of E2 was completely abolished by the addition of both estrogen receptor antagonists ICI 182,780 and G36 (Fig. 6).

3.4. GPR30 blockage partially offsets estrogen reduced casepase-3 activation

Using immunocytochemistry, we investigated the activation status of caspase-3, a marker of apoptosis, in resting and E2 stimulated NP cells. Immunostaining showed that cells cultured with medium alone for 48 h did not possess activated caspase-3 protein expression (Fig. 7). Activated caspase-3 levels were significantly increased in NP cells after stimulation with 20 ng IL-

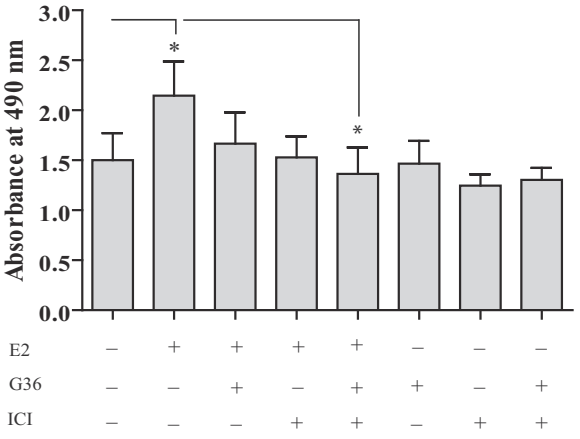

Fig. 4. E2 promoted cellular proliferation requires both ERs and GPR30. E2 treated intervertebral disc (IVD) cells showed significantly enhanced cellular proliferation when compared with control or co-treatment with G36 + ICI 182,780. Graph shows cell proliferation with the addition of E2 alone, E2 + G36, E2 + ICI 182,780, E2 + G36 + ICI 182,780, G36 or ICI 182,780 alone. * indicates $p \leq 0.05$ of comparison between E2 alone and control or between E2 alone and E2 + G36 + ICI 182,780. Data are presented as mean ± SE of triplicates in five independent samples.

1β, but simultaneous E2 exposure (in the presence of IL-1β) resulted in significantly reduced active caspase-3 protein staining. Pre-treatment with GPR30 receptor antagonist G36 partially restored caspase-3 staining in IL-1β-stimulated cells, and the presence of both E2 receptor antagonists G36 and ICI 182,780 almost completely blocked the E2 effect, showing levels of caspase-3 similar to that of IL-1β treatment alone (Fig. 7).

4. Discussion

GPR30 mediates estrogenic signals in various tissues, but its expression and localization in human intervertebral disc was not explored. In this study, expression of GPR30 in human fetal spine and adult IVD tissue was demonstrated for the first time. In the adult IVD samples, expression of GPR30 was observed in NP tissue and cultured NP cells at both mRNA and protein levels. GPR30 was expressed in degenerated disc tissue at similar levels to that of the scoliotic disc group. This suggests that, unlike the classical nuclear estrogen receptors ERα and ERβ [26], even degenerated disc NP cells may retain their potential to respond to estrogen through its receptor GPR30 for tissue regeneration.

Pervious work has demonstrated that the highest levels of GPR30 expression were observed in hypertrophic chondrocytes in the human growth plate and that the level of GPR30 expression declines as puberty progresses in both genders, suggesting that GPR30 may be involved in chondrogenesis [27]. Other studies performed on human bone tissue have shown that there is no sex or age difference in GPR30 protein expression during puberty [28]. Our study of disc NP tissues also detected no sex or age differences in GPR30 expression based on a small sample size. Since a relatively low number of female samples and a median age of 41 ± 15 indicative of a lack of female tissues of menopausal age, we cannot draw conclusions regarding GPR30 expression during menopause progression.

Estrogens play multiple protective roles in the musculoskeletal system [10,11,29]. GPR30 clearly contributes to estrogen signaling [25], with roles in glucose metabolism and skeletal bone growth reported [30–32]. The contradictory roles of GPR30 in the regulation of bone formation seem to result from gender specific effects. In knockout mouse models, deletion of GPR30 reduced bone growth in female mice [30,31], but increased bone growth in male mice [32]. It has also been shown that GPR30 is required for

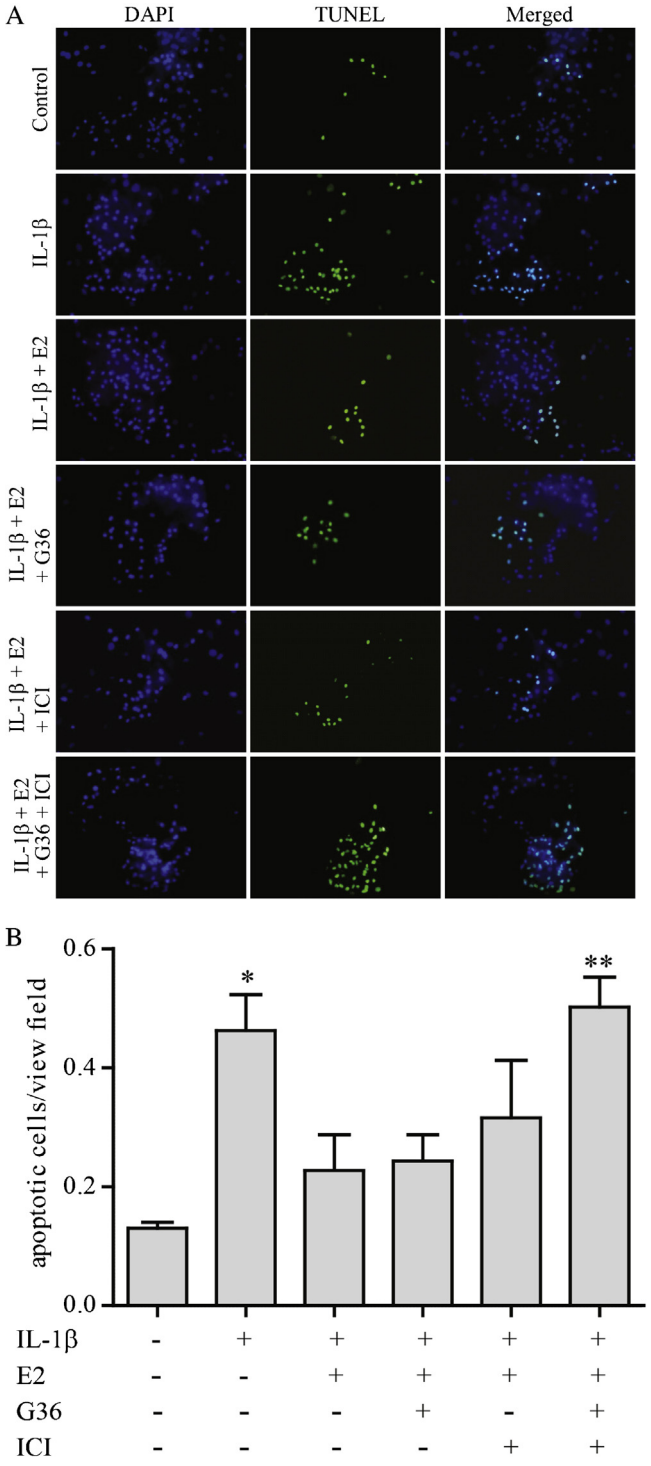

Fig. 5. Preventative effects of E2 on IL-1β induced disc cell apoptosis detected by TUNEL assay. (A) Florescent microscope images of cultured disc NP cells stained with either DAPI (blue) or TUNEL labeling (green) following treatment with either media alone (control), IL-1β, IL-1β + E2, IL-1β + E2 + G36, IL-1β + E2 + G36 + ICI 182,780. Positive TUNEL staining indicative of apoptosis was present in IL-1β-stimulated cell populations. Image magnification: ×100. (B) Quantitation of apoptotic disc cells (percentage of TUNEL positive cells) in the different treatment groups. Data are presented as mean + SE from three independent experiments ($n = 3$) (**$p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

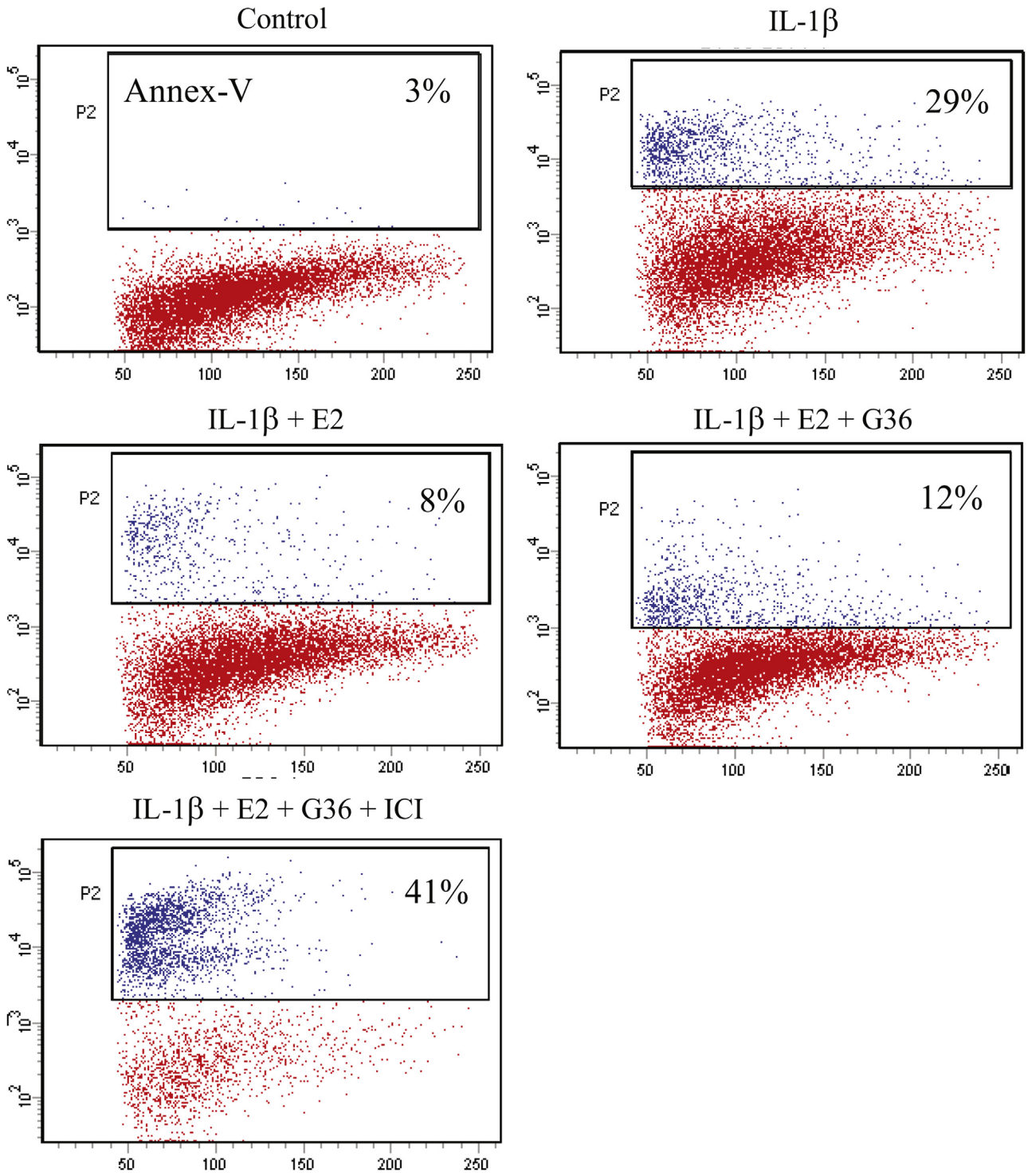

Fig. 6. Preventative effects of E2 on IL-1β-induced apoptosis in cultured disc NP cells detected by Alexa Fluor-488 conjugated annexin-V flow cytometry assay. Figure shows NP cells cultured in the presence of IL-1β, with the addition of combinations of E2, GPR30 antagonists G36 and/or ER antagonist ICI 182,780. The percentage of annexin-V positive cells is shown represented in the upper boxed region.

the estrogen induced self-renewal and regulation of primordial germ cell proliferation and fetal germ cell development [33]. We detected what appeared to be temporal expression of GPR30 in the developing human spine. Intracellular GPR30 expression was detected in the fetal spine NP at 12–14 weeks gestation but was absent or undetectable at 8–11 weeks. This may indicate a need for the specific signal transduction in the NP by GPR30 at that particular stage of spinal development. Further studies are needed to explore what specific purpose the expression of GPR30 expression in disc cells may convey and how this impacts on disc development.

In this study, we found that E2 could stimulate adult NP cells to proliferate, demonstrating a protective effect of E2 in this disc tissue. E2 induced cell proliferation was also shown in human

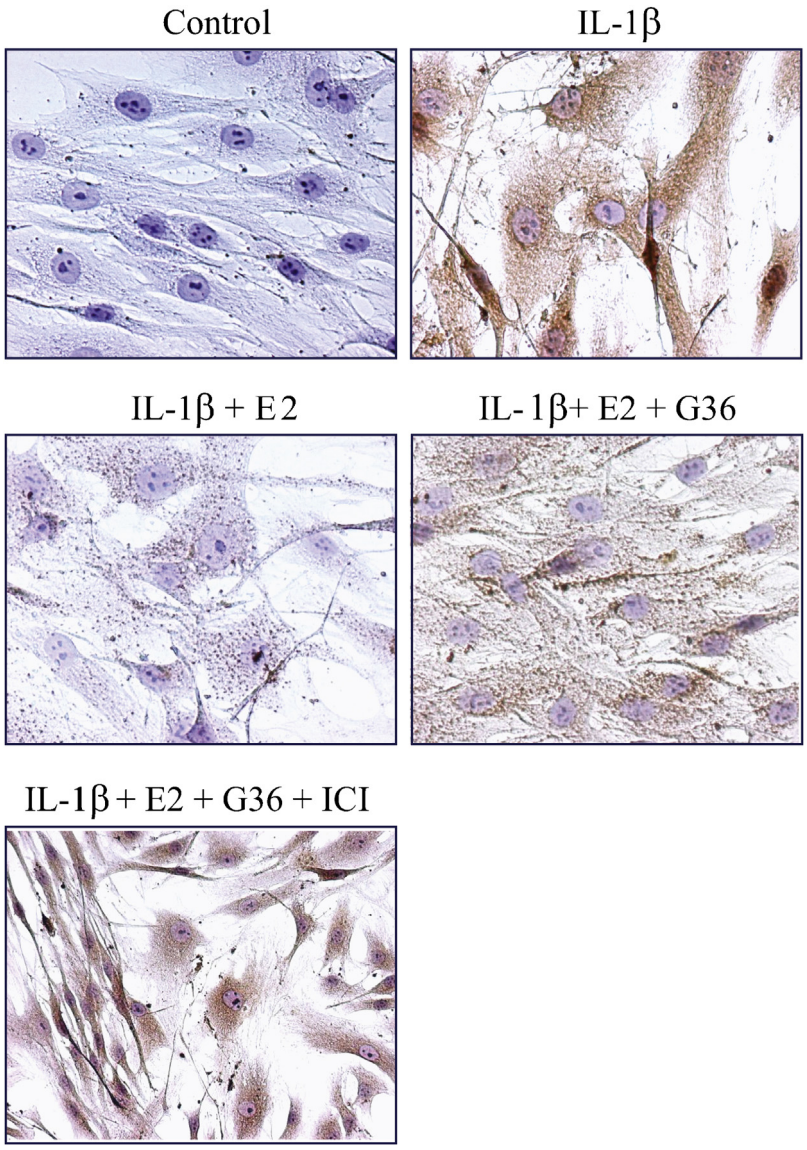

Fig. 7. Representative images of immunocytochemical staining of activated caspase-3 in disc NP cell cultures. Images show cultured NP cells treated with IL-1β and combinations of E2 alone, of E2 and specific estrogen receptor antagonists G36 and/or ICI 182,780, stained with specific anti-caspase-3 antibodies. Control shows unstimulated cells stained with anti-caspase-3 antibody. Image magnification: ×400.

annulus fibrosis cells [7], and this was correlated with cytoplasmic expression of ERβ. Similarly E2 stimulated disc cell proliferation in animal models [9] and expression of ERα/ERβ in human NP cellshas been confirmed [26]. Our current study demonstrated GPR30 expression in human NP tissue and cultured NP cells. When the NP cells were treated with either the GPR30 antagonist G36 or the ERα/ERβ antagonist ICI 182,780, E2 stimulated proliferation was partially suppressed, suggesting that both classic ER and GPR30 contribute to E2 signal transduction. There may be other roles for this receptor in the disc that are yet to be determined.

GPR30 has been previously localized to the plasma membrane where it mediates rapid estrogen signal transduction [34,35], and intracellular expression has also been reported in the endoplasmic reticulum [22] and in the nucleus [21] of various types of cells. We detected GPR30 protein expression in both plasma membrane and intracellular compartments of NP cells, but the expression was predominantly localized in the nucleus in all of the samples. Receptors in the family of G protein-coupled receptors are linked to non-genomic signal transduction mediated by complex intracellular pathways, often involving rapid calcium mobilization and cAMP elevation, a more rapid response to estrogen than the traditional nuclear receptors ERα and ERβ. While it remains unclear whether its intracellular expression is of functional significance [22] or is a result of intracellular trafficking, a recent study demonstrated that a nuclear localization signal was within the GPR30 protein sequence and an importin-dependent mechanism facilitated receptor translocation into the nucleus in breast cancer-associated fibroblasts [21]. Importantly, nuclear localization was functionally required, highlighting a potential alternative mechanism of E2 action through GPR30. In this study, we did not detect a rapid response to E2 stimulation in NP cells, rather the E2 mediated changes were observed after 24 h stimulation with or without ER inhibition. Although the E2 signal pathway through GPR30 remains largely unknown in disc cells, it can be postulated that GPR30 translocation into the nucleus was required for the cell proliferation and protective effects of E2, which may be via an importin-dependent mechanism.

IL-1β is an inflammatory cytokine that was previously shown to induce apoptosis in intervertebral disc cells [36,37]. We have also reported that IL-1β invokes apoptosis in human NP cell cultures, as evidenced by elevated levels of annexin V, caspase-3 activity and the induction of DNA fragmentation. In this study, the addition of E2 was able to protect NP cell populations from IL-1β induced cell death, consistent with reduced levels of caspase pathway intermediate caspase-3. These results are supported by previous observations where E2 was proven to protect rat NP cells against apoptosis induced by IL-1β [8], or by levofloxacin [9]. Interestingly, the effects of E2 on IL-1β-induced NP cell apoptosis were only partly blocked by GPR30 antagonist G36, but completely blocked by the combination of classic ER antagonists ICI 182,780 and G36. The data suggest that activation of GPR30 is necessary but not sufficient to mediate the full effect of E2 on IL-1β induced apoptosis. Both GPR30 and classic ER are involved in E2 mediated NP cell proliferation and survival. Caspases, belonging to a family of cysteine proteases, are key mediators of the apoptosis pathway. Caspase-3 is a downstream member of the caspase cascade and acts as a central effector in the apoptotic pathway [38]. E2 significantly inhibited caspase-3 activity triggered by IL-1β. These results indicate that anti-apoptotic effect of E2 on NP cells is, at least partly, mediated through a mechanism involving caspase-3 activation.

Several studies have shown both *in vitro* [22,35,39] and *in vivo* [40] that GPR30-specific agonists initiate multiple intracellular signaling pathways through G-protein intermediates. Signals include those mediated by changing levels of intracellular calcium ions, lipid and protein kinase pathway activation and trans-activation of membrane bound epidermal growth factor receptor signaling [25]. Our study adds to this body of work, by being the first to show that GPR30 is expressed in the human disc NP, can mediate E2 enhanced cell proliferation and influence cellular survival in disc cells, thus providing a potential avenue for promoting disc maintenance and repair. However, we realize a methodological limitation in using pharmacological inhibitors ICI 182,780 and G36 for loss of function experiments. The ICI 182,780 is also an agonist and while this confounds defining the exact mechanisms underlying the results, a role for GPR30 is clear [18]. Further studies are needed to identify the activation of intracellular pathways in IVD cells through GPR30 mediated estrogen signaling by gene silencing or knockout techniques.

Female menopause is associated with an increased risk of disc degeneration [14,41], which may indicate the contribution of estrogen withdrawal in the pathogenesis of disc degeneration disease. Similarly, the incidence of L4–L5 spondylolisthesis is also greater in women [42]. The effects of E2 mediated via GPR30 in disc NP cells may stimulate the development of potential regenerative strategies using specific GPR30 agonists or phytoestrogens that could reduce the side effects of hormone replacement therapy for women at risk of disc degenerative diseases.

Conflicts of interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

Acknowledgments

Funding for the study was provided initially by Cellixe (an established company) and then by internal grants from Spine Service.

References

[1] T. Vos, A.D. Flaxman, M. Naghavi, R. Lozano, C. Michaud, M. Ezzati, et al., Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010, Lancet 380 (2012) 2163–2196.
[2] S. Roberts, H. Evans, J. Trivedi, J. Menage, Histology and pathology of the human intervertebral disc, J. Bone Joint Surg. 88A (2006) 10–14.
[3] K.M. Cheung, J. Karppinen, D. Chan, D.W. Ho, Y.Q. Song, P. Sham, et al., Prevalence and pattern of lumbar magnetic resonance imaging changes in a population study of one thousand forty-three individuals, Spine (Phila Pa 1976) 34 (2009) 934–940.
[4] K. Singh, K. Masuda, E.J. Thonar, H.S. An, G. Cs-Szabo, Age-related changes in the extracellular matrix of nucleus pulposus and anulus fibrosus of human intervertebral disc, Spine (Phila Pa 1976) 34 (2009) 10–16.
[5] N.V. Vo, R.A. Hartman, T. Yurube, L.J. Jacobs, G.A. Sowa, J.D. Kang, Expression and regulation of metalloproteinases and their inhibitors in intervertebral disc aging and degeneration, Spine J. (2013).
[6] F. Ding, Z.W. Shao, L.M. Xiong, Cell death in intervertebral disc degeneration, Apoptosis 18 (2013) 777–785.
[7] H.E. Gruber, D. Yamaguchi, J. Ingram, K. Leslie, W. Huang, T.A. Miller, et al., Expression and localization of estrogen receptor-beta in annulus cells of the human intervertebral disc and the mitogenic effect of 17-beta-estradiol in vitro, BMC Musculoskelet. Disord. 3 (2002) 4.
[8] S.D. Yang, L. Ma, T.X. Gu, W.Y. Ding, F. Zhang, Y. Shen, et al., 17beta-estradiol protects against apoptosis induced by levofloxacin in rat nucleus pulposus cells by upregulating integrin alpha2beta1, Apoptosis 19 (2014) 789–800.
[9] H. Wang, W. Ding, D. Yang, T. Gu, S. Yang, Z. Bai, Different concentrations of 17beta-estradiol modulates apoptosis induced by interleukin-1beta in rat annulus fibrosus cells, Mol. Med. Rep. 10 (2014) 2745–2751.
[10] J.A. Gustafsson, What pharmacologists can learn from recent advances in estrogen signalling, Trends Pharmacol. Sci. 24 (2003) 479–485.
[11] N. Heldring, A. Pike, S. Andersson, J. Matthews, G. Cheng, J. Hartman, et al., Estrogen receptors: how do they signal and what are their targets, Physiol. Rev. 87 (2007) 905–931.
[12] J. Calleja-Agius, Y. Muscat-Baron, M.P. Brincat, Estrogens and the intervertebral disc, Menopause Int. 15 (2009) 127–130.
[13] J. Takatalo, J. Karppinen, J. Niinimaki, S. Taimela, S. Nayha, M.R. Jarvelin, et al., Prevalence of degenerative imaging findings in lumbar magnetic resonance imaging among young adults, Spine (Phila Pa 1976) 34 (2009) 1716–1721.
[14] Y.X. Wang, J.F. Griffith, Effect of menopause on lumbar disk degeneration: potential etiology, Radiology 257 (2010) 318–320.
[15] Y.M. Baron, M.P. Brincat, R. Galea, N. Calleja, Intervertebral disc height in treated and untreated overweight post-menopausal women, Hum. Reprod. 20 (2005) 3566–3570.
[16] T. Wang, L. Zhang, C. Huang, A.G. Cheng, G.T. Dang, Relationship between osteopenia and lumbar intervertebral disc degeneration in ovariectomized rats, Calcif. Tissue Int. 75 (2004) 205–213.
[17] K. Imada, H. Matsui, H. Tsuji, Oophorectomy predisposes to degenerative spondylolisthesis, J. Bone Joint Surg. Br. 77 (1995) 126–130.
[18] P. Thomas, Y. Pang, E.J. Filardo, J. Dong, Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells, Endocrinology 146 (2005) 624–632.
[19] B. Olde, L.M. Leeb-Lundberg, GPR30/GPER1: searching for a role in estrogen physiology, Trends Endocrinol. Metab. 20 (2009) 409–416.
[20] E.J. Filardo, P. Thomas, Minireview: G protein-coupled estrogen receptor-1 GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology, Endocrinology 153 (2012) 2953–2962.
[21] M. Pupo, A. Vivacqua, I. Perrotta, A. Pisano, S. Aquila, S. Abonante, et al., The nuclear localization signal is required for nuclear GPER translocation and function in breast Cancer-Associated Fibroblasts (CAFs), Mol. Cell Endocrinol. 376 (2013) 23–32.
[22] C.M. Revankar, D.F. Cimino, L.A. Sklar, J.B. Arterburn, E.R. Prossnitz, A transmembrane intracellular estrogen receptor mediates rapid cell signaling, Science 307 (2005) 1625–1630.
[23] C.G. Bologa, C.M. Revankar, S.M. Young, B.S. Edwards, J.B. Arterburn, A.S. Kiselyov, et al., Virtual and biomolecular screening converge on a selective agonist for GPR30, Nat. Chem. Biol. 2 (2006) 207–212.
[24] L. Albanito, A. Madeo, R. Lappano, A. Vivacqua, V. Rago, A. Carpino, et al., G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17beta-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells, Cancer Res. 67 (2007) 1859–1866.
[25] E.R. Prossnitz, J.B. Arterburn, H.O. Smith, T.I. Oprea, L.A. Sklar, H.J. Hathaway, Estrogen signaling through the transmembrane G protein-coupled receptor GPR30, Annu. Rev. Physiol. 70 (2008) 165–190.
[26] X.X. Song, Y.J. Yu, X.F. Li, Z.D. Liu, B.W. Yu, Z. Guo, Estrogen receptor expression in lumbar intervertebral disc of the elderly: gender- and degeneration degree-related variations, Joint Bone Spine 81 (2014) 250–253.
[27] A.S. Chagin, L. Savendahl, GPR30 estrogen receptor expression in the growth plate declines as puberty progresses, J. Clin. Endocrinol. Metab. 92 (2007) 4873–4877.
[28] T.J. Heino, A.S. Chagin, L. Savendahl, The novel estrogen receptor G-protein-coupled receptor 30 is expressed in human bone, J. Endocrinol. 197 (2008) R1–6.
[29] D. Somjen, Y. Weisman, A. Harell, E. Berger, A.M. Kaye, Direct and sex-specific stimulation by sex steroids of creatine kinase activity and DNA synthesis in rat bone, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 3361–3365.
[30] U.E. Martensson, S.A. Salehi, S. Windahl, M.F. Gomez, K. Sward, J. Daszkiewicz-Nilsson, et al., Deletion of the G protein-coupled receptor 30 impairs glucose

tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice, Endocrinology 150 (2009) 687–698.
[31] S.H. Windahl, N. Andersson, A.S. Chagin, U.E. Martensson, H. Carlsten, B. Olde, et al., The role of the G protein-coupled receptor GPR30 in the effects of estrogen in ovariectomized mice, Am. J. Physiol. Endocrinol. Metab. 296 (2009) E490–496.
[32] J. Ford, A. Hajibeigi, M. Long, L. Hahner, C. Gore, J.T. Hsieh, et al., GPR30 deficiency causes increased bone mass, mineralization, and growth plate proliferative activity in male mice, J. Bone Miner. Res. 26 (2011) 298–307.
[33] C. Ge, M. Yu, C. Zhang, G protein-coupled receptor 30 mediates estrogen-induced proliferation of primordial germ cells via EGFR/Akt/beta-catenin signaling pathway, Endocrinology 153 (2012) 3504–3516.
[34] T. Funakoshi, A. Yanai, K. Shinoda, M.M. Kawano, Y. Mizukami, G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane, Biochem. Biophys. Res. Commun. 346 (2006) 904–910.
[35] E. Filardo, J. Quinn, Y. Pang, C. Graeber, S. Shaw, J. Dong, et al., Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane, Endocrinology 148 (2007) 3236–3245.
[36] C.L. Le Maitre, A.J. Freemont, J.A. Hoyland, The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration, Arthritis Res. Ther. 7 (2005) R732–745.
[37] C.Q. Zhao, D. Liu, H. Li, L.S. Jiang, L.Y. Dai, Interleukin-1beta enhances the effect of serum deprivation on rat annular cell apoptosis, Apoptosis 12 (2007) 2155–2161.
[38] S. Shalini, L. Dorstyn, S. Dawar, S. Kumar, Old, new and emerging functions of caspases, Cell Death Differ. 22 (2015) 526–539.
[39] E.J. Filardo, P. Thomas, GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release, Trends Endocrinol. Metab. 16 (2005) 362–367.
[40] M.K. Dennis, R. Burai, C. Ramesh, W.K. Petrie, S.N. Alcon, T.K. Nayak, et al., In vivo effects of a GPR30 antagonist, Nat. Chem. Biol. 5 (2009) 421–427.
[41] C. Lou, H.L. Chen, X.Z. Feng, G.H. Xiang, S.P. Zhu, N.F. Tian, et al., Menopause is associated with lumbar disc degeneration: a review of 4230 intervertebral discs, Climacteric 17 (2014) 700–704.
[42] M.T. Vogt, D.A. Rubin, L. Palermo, L. Christianson, J.D. Kang, M.C. Nevitt, et al., Lumbar spine listhesis in older African American women, Spine J. 3 (2003) 255–261.